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TITLE OF THE INVENTION (280 characters max)

Improved Immunoassays

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Respectfully submitted,

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PATENT APPLICATION

IMPROVED IMMUNOASSAYS

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IMPROVED IMMUNOASSAYS

FIELD OF THE INVENTION

[01] The present invention relates to methods of improving tagged immunoassays.

BACKGROUND

[02] Immunoassays are used extensively in research in order to detect the presence of protein in a sample. Traditional immunoassays techniques, however suffer from many disadvantages. One factor that reduces the efficacy of immunoassays is an inability to characterize and accurately quantify the amount of protein present in the reagents. Another factor that reduces the efficacy of immunoassays in the inability to characterize and accurately quantify the different isoformic forms of the peptide that is ultimately detected. A proper characterization of quantification of the different isoformic forms of a particular peptide in a sample is essential, however, in order to accurately correlate the concentration of protein in a sample with a disease state. Accordingly, a need exists for improved immunoassay systems that provide for improved immunoassay systems that are capable of quantifying the quality and amount of protein present in sample.

SUMMARY

[03] Methods are described herein for immunoassay systems which are useful for qualifying an antibody in an antibody reagent for tagged immunoassay.

[04] In one aspect, a method is described which qualifies an antibody in an antibody reagent for tagged immunoassay by SELDI. In a further aspect, the method is used to qualify the antibody by determining the amount of antibody as a function of total protein. In a detailed aspect, the method further includes preparing antibody reagent in which the amount of antibody in the reagent comprises an amount reflected in the amount determined by SELDI.

[05] In another aspect, a method is described which qualifies peptides in a calibrator for tagged immunoassay by SELDI. In a further aspect, the method is used to qualify peptides by determining the amount of one or more particular peptides as a function of total protein. In a detailed aspect, the method further includes preparing an antibody reagent in which the amount

of antibody in the reagent comprises an amount reflected in the amount determined by SELDI. In a further aspect, the method includes qualifying the antibody in an antibody reagent for the tagged immunoassay using a SELDI immunoassay. In a detailed aspect, the tagged immunoassay is a BNP immunoassay. In a further detailed aspect, SELDI is SEAC. In a further detailed aspect, SELDI is SEND.

[06] In another aspect, a method is described which includes the steps of qualifying the polypeptides captured by an antibody reagent in a tagged immunoassay by providing a SELDI probe comprising the antibody reagent attached to a surface of the probe, contacting the antibody reagent with a sample, whereby the antibody reagent captures polypeptides from the sample, and detecting the captured polypeptides by SELDI. In a detailed aspect, the tagged immunoassay is a BNP immunoassay. In a further detailed aspect, SELDI is SEAC. In a further detailed aspect, SELDI is SEND.

[07] In another aspect, a method is described which includes specifically detecting at least one BNP isoform by SELDI immunoassay. In a further aspect, the BNP isoform is selected from BNP^{77-108}, BNP^{77-106}, BNP^{39-86}, BNP^{53-85}, BNP^{66-98}, BNP^{30-106}, BNP^{11-107}, BNP^{9-106}, BNP^{69-100}, and BNP^{76-107}.

[08] In another aspect, a method is described which includes preparing a calibrant for a BNP tagged immunoassay comprising mixing plasma, BNP and at least one protease inhibitor. In a further aspect, the at least one protease inhibitor is a plurality of protease inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

[09] FIG. 1. Predicted amino acid sequence of B-type Natriuretic Peptide (BNP) Precursor and fragments thereof is shown. Fragment Arg77– His108 (indicated on the figure as "77-108") is one isoform sought to be detected by immunoassay.

[10] FIG. 2A and B. Mass spectra of proteins in a BNP immunoassay calibrator solution. SELDI analysis of a calibrator used for BNP immunoassays demonstrates that the calibrator contains many polypeptides besides full length BNP (BNP^{77-108}). The peak at 3464 corresponds to BNP^{77-108}. The peak at 66283.6 presumably corresponds to bovine serum albumin.

[11] Fig. 3. Mass spectrum of antibody reagent comprising anti-BNP monoclonal also contains peaks corresponding to many proteins besides the antibody.

[12] Fig. 4A, B, C, and D. Mass spectra of proteins from a BNP calibrator solution captured by SELDI immunoassay. Proteins from the calibrator were spiked into human plasma. Anti-BNP was used to capture the proteins. Besides the 77-108 isoform at 6461, peaks are detected whose molecular weights correspond to BNP peptide fragments: A BNP isoform that weighs

about 3170.8 Da and corresponds to amino acids 77 to 106 of proBNP; a BNP isoform that weighs about 3280 Da and corresponds to amino acid 79 to 108 of proBNP; a BNP isoform that weighs about 3671 Da and corresponds to amino acid 53-85 (3669) or 66-98 (3674.4) of proBNP; a BNP isoform that weighs about 8215.5 Da and corresponds to amino acids 30 to 103 of proBNP; a BNP isoform that weighs about 10875.3 and corresponds to 11-107 (108755.) or 9-106 (10874.4)of proBNP.

[13] Fig. 5A and B. Mass spectra and standard curve of BNP calibrator at various levels of concentration. Spectra show that the calibrator contains as much BNP79-108 isoform as BNP77-108 isoform.

[14] Fig. 6A, B and C. Mass spectra and standard curve of BNP calibrator at various levels of concentration. BNP^{77-108} is hardly visible. When the standard is calibrated to the amount of protein corresponding to BNP^{79-106}, BNP^{79-108} and a peak corresponding to either BNP^{69-100} or BNP^{76-107} the standard curve is skewed to the right, implying that a test measurement contains more BNP that the original calibrator key indicated.

[15] Fig. 7A and B. Mass spectra of subject samples. Peaks corresponding to BNP^{77-109} are difficult to detect. However, degraded forms of BNP appear to be present – about 3152 (BNP^{77-106}?) and about 3282 (BNP^{79-108}?).

DETAILED DESCRIPTION

[16] "Probe" in the context of this invention refers to a device adapted to engage a probe interface of a gas phase ion spectrometer (e.g., a mass spectrometer) and to present an analyte to ionizing energy for ionization and introduction into a gas phase ion spectrometer, such as a mass spectrometer. A "probe" will generally comprise a solid substrate (either flexible or rigid) comprising a sample presenting surface on which an analyte is presented to the source of ionizing energy.

[17] "Surface-enhanced laser desorption/ionization" or "SELDI" refers to a method of desorption/ionization gas phase ion spectrometry (e.g., mass spectrometry) in which the analyte is captured on the surface of a SELDI probe that engages the probe interface of the gas phase ion spectrometer. In "SELDI MS," the gas phase ion spectrometer is a mass spectrometer. SELDI technology is described in, e.g., U.S. patent 5,719,060 (Hutchens and Yip) and U.S. patent 6,225,047 (Hutchens and Yip).

[18] "Surface-Enhanced Affinity Capture" or "SEAC" is a version of SELDI that involves the use of probes comprising an absorbent surface (a "SEAC probe"). "Adsorbent surface" refers to a surface to which is bound an adsorbent (also called a "capture reagent" or an "affinity

reagent"). An adsorbent is any material capable of binding an analyte (e.g., a target polypeptide or nucleic acid). "Chromatographic adsorbent" refers to a material typically used in chromatography. Chromatographic adsorbents include, for example, ion exchange materials, metal chelators (e.g., nitriloacetic acid or iminodiacetic acid), immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, simple biomolecules (e.g., nucleotides, amino acids, simple sugars and fatty acids) and mixed mode adsorbents (e.g., hydrophobic attraction/electrostatic repulsion adsorbents). "Biospecific adsorbent" refers an adsorbent comprising a biomolecule, e.g., a nucleic acid molecule (e.g., an aptamer), a polypeptide, a polysaccharide, a lipid, a steroid or a conjugate of these (e.g., a glycoprotein, a lipoprotein, a glycolipid, a nucleic acid (e.g., DNA)-protein conjugate). In certain instances the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Examples of biospecific adsorbents are antibodies, receptor proteins and nucleic acids. Biospecific adsorbents typically have higher specificity for a target analyte than chromatographic adsorbents. Further examples of adsorbents for use in SELDI can be found in U.S. Patent 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001).

[19] In some embodiments, a SEAC probe is provided as a pre-activated surface which can be modified to provide an adsorbent of choice. For example, certain probes are provided with a reactive moiety that is capable of binding a biological molecule through a covalent bond. Epoxide and carbodiimidizole are useful reactive moieties to covalently bind biospecific adsorbents such as antibodies or cellular receptors.

[20] "Adsorption" refers to detectable non-covalent binding of an analyte to an adsorbent or capture reagent.

[21] "Surface-Enhanced Neat Desorption" or "SEND" is a version of SELDI that involves the use of probes comprising energy absorbing molecules chemically bound to the probe surface. ("SEND probe.") "Energy absorbing molecules" ("EAM") refer to molecules that are capable of absorbing energy from a laser desorption/ ionization source and thereafter contributing to desorption and ionization of analyte molecules in contact therewith. The phrase includes molecules used in MALDI , frequently referred to as "matrix", and explicitly includes cinnamic acid derivatives, sinapinic acid ("SPA"), cyano-hydroxy-cinnamic acid ("CHCA") and dihydroxybenzoic acid, ferulic acid, hydroxyacetophenone derivatives, as well as others. It also includes EAMs used in SELDI. SEND is further described in United States patent 5,719,060 and United States patent application 60/408,255, filed September 4, 2002 (Kitagawa, "Monomers

And Polymers Having Energy Absorbing Moieties Of Use In Desorption/Ionization Of Analytes").

[22] "Surface-Enhanced Photolabile Attachment and Release" or "SEPAR" is a version of SELDI that involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, e.g., laser light. SEPAR is further described in United States patent 5,719,060.

[23] "Eluant" or "wash solution" refers to an agent, typically a solution, which is used to affect or modify adsorption of an analyte to an adsorbent surface and/or remove unbound materials from the surface. The elution characteristics of an eluant can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength and temperature.

[24] "Analyte" refers to any component of a sample that is desired to be detected. The term can refer to a single component or a plurality of components in the sample.

[25] The "complexity" of a sample adsorbed to an adsorption surface of an affinity capture probe means the number of different protein species that are adsorbed.

[26] "Molecular binding partners" and "specific binding partners" refer to pairs of molecules, typically pairs of biomolecules that exhibit specific binding. Molecular binding partners include, without limitation, receptor and ligand, antibody and antigen, biotin and avidin, and biotin and streptavidin.

[27] "Monitoring" refers to recording changes in a continuously varying parameter.

[28] "Solid support" refers to a solid material which can be derivatized with, or otherwise attached to, a chemical moiety, such as a capture reagent, a reactive moiety or an energy absorbing species. Exemplary solid supports include chips (e.g., probes), microtiter plates and chromatographic resins.

[29] "Chip" refers to a solid support having a generally planar surface to which a chemical moiety can be attached. Chips that are adapted to engage a probe interface are also called "probes."

[30] "Biochip" refers to a chip to which a chemical moiety is attached. Frequently, the surface of the biochip comprises a plurality of addressable locations, each of which location has the chemical moiety attached there.

[31] "Protein biochip" refers to a biochip adapted for the capture of polypeptides. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems (Fremont, CA), Packard BioScience Company (Meriden CT), Zyomyx (Hayward, CA) and Phylos (Lexington, MA). Examples of such protein biochips are described

in the following patents or patent applications: U.S. patent 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001); International publication WO 99/51773 (Kuimelis and Wagner, "Addressable protein arrays," October 14, 1999); U.S. patent 6,329,209 (Wagner et al., "Arrays of protein-capture agents and methods of use thereof," December 11, 2001) and International publication WO 00/56934 (Englert et al., "Continuous porous matrix arrays," September 28, 2000).

[32] Protein biochips produced by Ciphergen Biosystems comprise surfaces having chromatographic or biospecific adsorbents attached thereto at addressable locations. Ciphergen ProteinChip® arrays include NP20, H4, H50, SAX-2, Q-10, WCX-2, CM-10, IMAC-3, IMAC-30, LSAX-30, LWCX-30, IMAC-40, PS-10, PS-20 and PG-20. These protein biochips comprise an aluminum substrate in the form of a strip. The surface of the strip is coated with silicon dioxide.

[33] In the case of the NP-20 biochip, silicon oxide functions as a hydrophilic adsorbent to capture hydrophilic proteins.

[34] H4, H50, SAX-2, Q-10, WCX-2, CM-10, IMAC-3, IMAC-30, PS-10 and PS-20 biochips further comprise a functionalized, cross-linked polymer in the form of a hydrogel physically attached to the surface of the biochip or covalently attached through a silane to the surface of the biochip. The H4 biochip has isopropyl functionalities for hydrophobic binding. The H50 biochip has nonylphenoxy-poly(ethylene glycol)methacrylate for hydrophobic binding. The SAX-2 and Q-10 biochips have quaternary ammonium functionalities for anion exchange. The WCX-2 and CM-10 biochips have carboxylate functionalities for cation exchange. The IMAC-3 and IMAC-30 biochips have nitriloacetic acid functionalities that adsorb transition metal ions, such as Cu^{++} and Ni^{++}, by chelation. These immobilized metal ions allow adsorption of peptide and proteins by coordinate bonding. The PS-10 biochip has carboimidizole functional groups that can react with groups on proteins for covalent binding. The PS-20 biochip has epoxide functional groups for covalent binding with proteins. The PS-series biochips are useful for binding biospecific adsorbents, such as antibodies, receptors, lectins, heparin, Protein A, biotin/streptavidin and the like, to chip surfaces where they function to specifically capture analytes from a sample. The PG-20 biochip is a PS-20 chip to which Protein G is attached. The LSAX-30 (anion exchange), LWCX-30 (cation exchange) and IMAC-40 (metal chelate) biochips have functionalized latex beads on their surfaces. Such biochips are further described in: WO 00/66265 (Rich et al., "Probes for a Gas Phase Ion Spectrometer," November 9, 2000); WO 00/67293 (Beecher et al., "Sample Holder with Hydrophobic Coating for Gas Phase Mass Spectrometer," November 9, 2000); U.S. patent application US 2003 0032043 A1 (Pohl and

Papanu, "Latex Based Adsorbent Chip," July 16, 2002) and U.S. patent application 60/350,110 (Um et al., "Hydrophobic Surface Chip," November 8, 2001); U.S. patent application 60/367,837, (Boschetti et al., "Biochips With Surfaces Coated With Polysaccharide-Based Hydrogels," May 5, 2002) and U.S. patent application entitled "Photocrosslinked Hydrogel Surface Coatings" (Huang et al., filed February 21, 2003).

[35] Upon capture on a biochip, analytes can be detected by a variety of detection methods selected from, for example, a gas phase ion spectrometry method, an optical method, an electrochemical method, atomic force microscopy and a radio frequency method. Gas phase ion spectrometry methods are described herein. Of particular interest is the use of mass spectrometry and, in particular, SELDI. Optical methods include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Immunoassays in various formats (e.g., ELISA) are popular methods for detection of analytes captured on a solid phase. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy.

[36] "Antibody" refers to a polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which specifically binds and recognizes an epitope (e.g., an antigen). This includes intact immunoglobulins and the variants and portions of them well known in the art such as, Fab' fragments, F(ab)'2 fragments, and scFv proteins. An scFv protein is a fusion protein in which a light chain variable region and a heavy chain variable region bound by a linker. Natural immunoglobulins are encoded by immunoglobulin genes. These include the kappa and lambda light chain constant region genes, the alpha, gamma, delta, epsilon and mu heavy chain constant region genes, and the myriad immunoglobulin variable region genes. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies and humanized antibodies, produced by immunization, from hybridomas, or recombinantly.

[37] "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH1, CH2 and CH3, but does not include the heavy chain variable region.

[38] "Epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by

tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols in METHODS IN MOLECULAR BIOLOGY, Vol. 66, Glenn E. Morris, Ed (1996).

[39] A ligand or a receptor "specifically binds to" a compound analyte when the ligand or receptor functions in a binding reaction which is determinative of the presence of the analyte in a sample of heterogeneous compounds. Thus, the ligand or receptor binds preferentially to a particular analyte and does not bind in a significant amount to other compounds present in the sample. For example, a polynucleotide specifically binds to an analyte polynucleotide comprising a complementary sequence and an antibody specifically binds under immunoassay conditions to an antigen analyte bearing an epitope against which the antibody was raised.

[40] "Immunoassay" refers to a method of detecting an analyte in a sample in which specificity for the analyte is conferred by the specific binding between an antibody and a ligand. This includes detecting an antibody analyte through specific binding between the antibody and a ligand. See Harlow and Lane (1988) ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. A "tagged immunoassay" is an immunoassay in which the analyte is not detected directly, but rather through detection of a tag or label. Generally, the analyte is itself tagged, or the immunoassay involved binding of the analyte with a tagged antibody which is, itself, tagged. The techniques of immunoassay using labeled reagents for detecting antigens and antibodies are sensitive. Solid-phase assays for antibodies employing ligands labeled with radioisotopes or enzymes (radioimmune assay; RIA and enzyme-linked immunosorbent assay; ELISA) are widely used because large numbers can be performed in a relatively short time. RIA and ELIZA are direct binding assays for antibody (or antigen) and both work on the same principle, but the means of detecting specific binding is different. For both methods, a pure preparation of a known antigen or antibody, or both, is needed in order to standardize the assay. In RIA for an antigen, pure antibody against that antigen is radioactively labeled, usually with ^{125}I; for the ELISA, an enzyme is linked chemically to the antibody. The unlabeled component, which in this case would be antigen, is attached to a solid support, such as the wells of a plastic multiwell plate, which will adsorb a certain amount of any protein. The labeled antibody is allowed to bind to the unlaeled antigen, under conditions where nonspecific adsorption is blocked, and any unbound antibody and other proteins are washed way. Antibody binding in RIA is measured directly in terms of the amount of

radioactivity retained by the coated wells, whereas in ELISA, binding is detected by a reaction that converts a colorless substrate into a colored reaction product. Labeled anti-immunoglobulin antibodies can also be used with RIA or ELISA to detect binding of unlabeled antibody to unlabeled antigen-coated plates.

[41] A modification of ELISA known as a "capture" or "sandwich ELISA" (or more generally referred to as an "antigen-capture assay") can be used to detect secreted products such as cytokines. Rather than the antigen being directly attached to a plastic plate, antigen-specific antibodies are bound to the plate. These are able to bind antigen with high affinity, and thus concentrate it on the surface of the plate, even with antigens that are present in very low concentrations in the initial mixture. A separate labeled antibody that recognizes a different epitope to the immobilized first antibody is then used to detect the bound antigen.

[42] RIA and ELISA do not allow one to measure directly the amount of antigen or antibody in a sample of unknown composition, as both depend on the binding of a pure labeled antigen or antibody. In a "competitive inhibition assay," the presence and amount of a particular antigen in an unknown sample is determined by its ability to compete with a labeled reference antigen for binding to an antibody typically attached to a plastic well. A standard curve is first constructed by adding varying amounts of a known, unlabeled standard preparation; the assay can then measure the amount of antigen in unknown samples by comparison with the standard. The competitive binding assay can also be used for measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody.

[43] "Detectable moiety" or a "label" or a "tag" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, ^{35}S, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin-streptavadin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantify the amount of bound detectable moiety in a sample. The detectable moiety can be incorporated in or attached to a primer or probe either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., incorporation of radioactive nucleotides, or biotinylated nucleotides that are recognized by streptavadin. The detectable moiety can be directly or indirectly detectable. Indirect detection can involve the binding of a second directly or indirectly detectable moiety to the detectable moiety. For example, the detectable moiety can be the ligand of a binding partner, such as

biotin, which is a binding partner for streptavadin, or a nucleotide sequence, which is the binding partner for a complementary sequence, to which it can specifically hybridize. The binding partner can itself be directly detectable, for example, an antibody can be itself labeled with a fluorescent molecule. The binding partner also can be indirectly detectable, for example, a nucleic acid having a complementary nucleotide sequence can be a part of a branched DNA molecule that is in turn detectable through hybridization with other labeled nucleic acid molecules. (See, e.g., P. D. Fahrlander and A. Klausner, Bio/Technology 6:1165 (1988)). Quantitation of the signal is achieved by, e.g., scintillation counting, densitometry, or flow cytometry.

IMPROVING IMMUNOASSAYS

[44] The present invention provides improved methods of tagged immunoassay. In particular, the present invention relates to using mass spectrometry, and in particular, SELDI, for improving traditional immunoassay techniques.

[45] In one aspect, this invention provides a method for characterizing and providing quality control for the antibody reagent used in an immunoassay, e.g., a tagged immunoassay. It has been found that antibody reagents used in immunoassay kits can contain contaminating proteins. These contaminants can interfere with the measurement of the actual amount of antibodies in the practitioner regarding how much antibody reagent to use. Methods are useful for qualify control in the preparation and use of antibody reagents. The methods involve measuring the amount of antibody and/or the amounts of other proteins in an antibody reagent for use in an immunoassay, e.g., in a tagged immunoassay kit. The antibody can be qualified both in terms the amount of the antibody and its quality, e.g., its state of degradation. Reagents that do not pass quality control standards for any qualifier of interest can be discarded or modified to come into compliance. Instructions for use of the reagent can take into consideration the quality of the reagent and the impact of this quality on the immunoassay. For example, one generally wants to use enough antibody reagent to capture all the target protein of interest in a sample, Therefore, the amount of antibody included in an antibody reagent can be determined with reference to the amount measured by mass spectrometry, e.g., SELDI compared with, e.g., total protein.

[46] In addition, the kits can include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g.,

magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media can include addresses to internet sites that provide such instructional materials.

[47] In another aspect, this invention provides a more precise assay for BNP. Human B-type natiuretic peptide is a member of the cardiac natriuretic peptide family. It is a 32 amino acid peptide with potent natiuretic, diuretic, and vasodilatory endocrine functions. Expression of the BNP gene is predominantly in the myocytes of the failing heart. Patients with congestive heart failure has increased amount of BNP secreted into circulation. Therefore accurate measurement of plasma BNP is valuable in the primary diagnosis of heart failure (Clerico, Sagnella, Mair, Maisel, Goetze).

[48] In immunoassays, the antibody reagent may recognize an epitope that exists not only in the target protein, but in degradation fragments of the target protein as well. For example, anti-BNP antibody recognizes not only BNP^{77-108} but degradation fragments of this protein as well. Traditional tagged immunoassays that employ such antibody reagents cannot distinguish between the various forms of the target protein.

[49] Furthermore, calibrators used in standard immunoassays may comprise not only full length calibrator protein, but degradation products, as well. This means that the calibrator may lead to mis-measurement of the amount of target in a sample. In fact, examination of a calibrant used for BNP immunoassays demonstrated that the calibrant contained not only full length BNP, but various degradation fragments of BNP, identifiable because their molecular weight corresponded to the molecular weight of identifiable sub-sequences of the BNP amino acid sequence.

[50] Accordingly, in one aspect this invention provides a method for providing quality control in the manufacture and use of immunoassay calibrators in general and BNP immunoassay calibrators in particular. In one embodiment, the method involves qualifying the peptides in an immunoassay calibrator, e.g., a BNP immunoassay calibrator, by mass spectrometry, in particular by SELDI. This method allows more precise discrimination of those peptides, as they can be both discriminated according to mass and quantified based on the area under a mass spectrum peak. According to the method, an immunoassay calibrator solution is characterized by mass spectrometry, in particular by SELDI. The differentiation and quantitation of the peptides is performed by mass spectrometry. In one version, the peptides are captured on an SELDI MS probe, such as a probe with a hydrophobic surface or a reactive probe derivatized with an antibody that specifically recognizes polypeptides with an epitope of the calibrator polypeptide. In particular the polypeptides in the calibrator can be captured on a probe derivatized with antibody reagent used in the immunoassay kit. One may then calibrate the assay

based on one or more of the peaks of interest. For example, the polypeptide can be measured as function of total protein in the calibrator.

[51] For example, in a BNP assay, the assay can be calibrated against BNP^{77-108}. In the case of a BNP assay, mass spectra showed that the calibrator in plasma contained many degraded forms of BNP. This implies the presence of proteases. Accordingly, one can stabilize the BNP polypeptide in the calibrator by adding one or more protease inhibitors.

[52] The antibody reagent in an immunoassay may not distinguish between a target polypeptide and degraded forms of a target polypeptide. Insofar as only one or some of these detected polypeptides may be responsible for the sensitivity and specificity of a diagnostic or other assay based on this detection, the detection of other polypeptides can impair sensitivity and specificity. Therefore, one may improve the assay by determining what other polypeptides are captured by the antibody reagent, and directing the assay to the detection, or use or specific polypeptides. In one embodiment, this may involve performing the assay as a sandwich assay in which the labeled antibody detects the isoform specified. Alternatively, the immunoassay may be a SELDI MS immunoassay. An immunoassay based on mass spectrometry automatically provides discrimination of the various captured polypeptides based on mass.

[53] In the case of BNP, while such immunoassays are directed to full length BNP, they detect other forms of BNP also. It is not known what impact the detection of these other forms has on the specificity or sensitivity of a diagnostic test that uses BNP to diagnose heart disease. However, the general target of these assays is BNP^{77-108}. Accordingly, on can perform a SELDI immunoassay in which the amount of BNP^{77-108} is measured. (Other fragments may be specifically detected if desired.) Alternatively, one can develop an antibody that is specific for BNP^{77-108}, and employ this in a sandwich tagged immunoassay.

[54] BNP is the C-terminal region of a 108 amino acid proBNP. proBNP comprises 108 amino acids and is represented by SEQ ID NO:1. This proBNP contains multiple sites for possible amino acid modifications and endoproteolytic cleavage. In fact, many different isoforms of BNP are found to circulate in plasma. Isoforms of BNP include, but are not limited to a BNP isoform that weighs about 3464 Da and corresponds to amino acid 77 to 108 of proBNP; a BNP isoform that weighs about 3280 Da and corresponds to amino acid 79 to 108 of proBNP; a BNP isoform that weighs about 3170.8 Da and corresponds to amino acids 77 to 106 of proBNP; a BNP isoform that weighs about 5377.3 Da and corresponds to amino acids 39 to 86 of proBNP; a BNP isoform that weighs about 3660 Da and corresponds to amino acids 53 to 85 of proBNP; a BNP isoform that weighs about 3674.4 Da and corresponds to amino acids 66 to 98 of proBNP; a BNP isoform that weighs about 8215.5 Da and corresponds to amino acids 30 to

103 of proBNP; a BNP isoform that weighs about 10875.5 Da and corresponds to amino acids 11 to 107 of proBNP; a BNP isoform that weighs about 10877.4 Da and corresponds to amino acids 9 to 106 of proBNP. Other isoforms of BNP are detectable by the methods of the present invention.

[55] Accordingly, in one embodiment, this invention provides methods for qualifying at least one form of a BNP polypeptide in a sample. The method comprises first providing a SELDI probe whose surface has been derivatized with antibodies that specifically bind to an epitope of BNP, preferably mature BNP. The probe can be a probe with a reactive surface, such as those described above. Such a probe is capable of specifically capturing the forms of BNP that comprise this epitope. Then, a sample for testing, such as a subject sample in a diagnostic test, is contacted with the bound antibodies. Polypeptides that possess the epitope are captured by the bound antibodies and unbound material is washed away. An energy absorbing molecule is then associated with the bound material. This may involve application of a traditional matrix. Alternatively, if the probe is a SEND probe on which energy absorbing molecules are already bound, no external matrix is necessary. The captured molecules are then detected by mass spectrometry. Because mass spectrometry qualifies analytes by mass, polypeptides comprising the same epitope, but differing in mass may be detected, differentiated and measured. For example, the amount of BNP^{77-108} can be differentiated from other forms of the molecule and quantified by this SELDI immunoassay. Indeed, examination of subject samples demonstrated that the antibody reagent used in BNP tagged immunoasays bound to many other species as well. The present invention allows the differentiation of these species.

[56] Traditional methods of detecting BNP in a sample are limited by their ability to detect total concentration of all BNP present in a sample. The present invention provides methods for detecting and quantifying the different isoforms of BNP present in a sample.

EXAMPLES

Example 1

[57] Anti-BNP-106.3 (monoclonal), anti-BNP-.5 (omniclonal) antibodies are supplied by Biosite. The antibodies were diluted to a final concentration of 0.5 mg/ml with 0.1M sodium bicarbonate 0.05% TritonX100 pH 9. Aliquots of 3 ul were added per spot of Reactive Surface (RS) ProteinChip array (Ciphergen). The coupling was allowed to proceed at 4C for 16 hr. The chips were blocked with 1M TrisHCl pH 8 and then BSA (1mg/ml) in 0.5M TrisHCl, 0.1% TritonX100 pH 8. Excess antibodies were washed away with 1% TritonX100 PBS, followed by 10% PEG 0.1% TritonX100 PBS and finally with 0.1% TritonX100 PBS.

[58] Purified BNP (Biosite) was diluted into 50% human serum (Intergen), or 50% human EDTA plasma (Biosite) with/without protease inhibitor cocktail (Roche). Aliquots of 100 ul of each BNP standard were incubated with antibodies immobilized on RS ProteinChip array in a bioprocessor (Ciphergen). BNP calibrators in plasma (Biosite) were diluted 1:1 and aliquots of 150 ul were incubated separately with antibodies on RS ProteinChip array. Chest pain patient EDTA plasma samples were diluted 1:1 and aliquots of 150 ul were incubated separately with antibodies on RS ProteinChip array. After 16 hr of incubation at 4C with shaking, the arrays were washed with 125 ul of 1M urea 0.1% CHAPS 50mM TrisHCl pH 7.5 two times. After rinsing with water and air dried, 2 ul of sinapinic acid or cyano hydroxycinnamic acid were added per spot. The retained proteins were detected by PBSII mass spectrometer (Ciphergen).

[59] The present invention has been described in detail, including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of the present disclosure, may make modifications and/or improvements of this invention and still be within the scope and spirit of this invention as set forth in the following claims.

[60] All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

What is Claimed:

1. A method comprising qualifying the antibody in an antibody reagent for tagged immunoassay by SELDI.

2. The method of claim **1** wherein qualifying comprises determining the amount of antibody as a function of total protein.

3. The method of claim **2** further comprising preparing antibody reagent in which the amount of antibody in the reagent comprises an amount reflected the amount determined by SELDI.

4. A method comprising qualifying the peptides in a calibrator for tagged immunoassay by SELDI.

5. The method of claim **[05]** wherein qualifying comprises determining the amount of one or more particular peptides as a function of total protein.

6. The method of claim **5** further comprising preparing antibody reagent in which the amount of antibody in the reagent comprises an amount reflected the amount determined by SELDI.

7. The method of claim **[05]** further comprising qualifying the antibody in an antibody reagent for the tagged immunoassay using a SELDI immunoassay

8. A method comprising the steps of qualifying the polypeptides captured by an antibody reagent in a tagged immunoassay by:

(a) providing a SELDI probe comprising the antibody reagent attached to a surface of the probe;

(b) contacting the antibody reagent with a sample, whereby the antibody reagent captures polypeptides from the sample; and

(c) detecting the captured polypeptides by SELDI.

9. The method of any of claims **1** to **8** wherein the tagged immunoassay is a BNP immunoassay.

10. The method of any of claims **1** to **8** wherein SELDI is SEAC.

11. The method of any of claims **1** to **8** wherein SELDI is SEND.

12. A method comprising specifically detecting at least one BNP isoform by SELDI immunoassay.

13. The method of claim [07] wherein the isoform is selected from BNP^{77-108}, BNP^{77-106}, BNP^{39-86}, BNP^{53-85}, BNP^{66-98}, BNP^{30-106}, BNP^{11-107}, BNP^{9-106}, BNP^{69-100}, and BNP^{76-107}.

14. A method comprising preparing a calibrant for a BNP tagged immunoassay comprising mixing plasma, BNP and at least one protease inhibitor.

15. The method of claim 14 wherein the at least one protease inhibitor is a plurality of protease inhibitors.

ABSTRACT

The present invention relates to methods of improving tagged immunoassays.

B-type Natriuretic Peptide (BNP) Precursor

His-Pro-Leu-Gly-Ser-Pro-Gly-Ser-Ala-Ser-Asp-Leu-Glu-Thr-Ser-Gly-Leu-Gln-Glu-Gln-Arg-Asn-His-Leu-Gln-Gly-Lys-Leu-Ser-Glu-Leu-Gln-Val-Glu-Gln-Thr-Ser-Leu-Glu-Pro-Leu-Gln-Glu-Ser-Pro-Arg-Pro-Thr-Gly-Val-Trp-Lys-Ser-Arg-Glu-Val-Ala-Thr-Glu-Gly-Ile-Arg-Gly-His-Arg-Lys-Met-Val-Leu-Tyr-Thr-Leu-Arg-Ala-Pro-Arg-Ser-Pro-Lys-Met-Val-Gln-Gly-Ser-Gly-Cys-Phe-Gly-Arg-Lys-Met-Asp-Arg-Ile-Ser-Ser-Ser-Ser-Gly-Leu-Gly-Cys-Lys-Val-Leu-Arg-Arg-His-

108

1-108		11903.6 Da	pI 10.6
77-108	BNP -32	3464.1 Da	pI 11.4
1-21		2166.3 Da	pI 4.5

FIG. 1

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Attorney: Leslie E. Aberman
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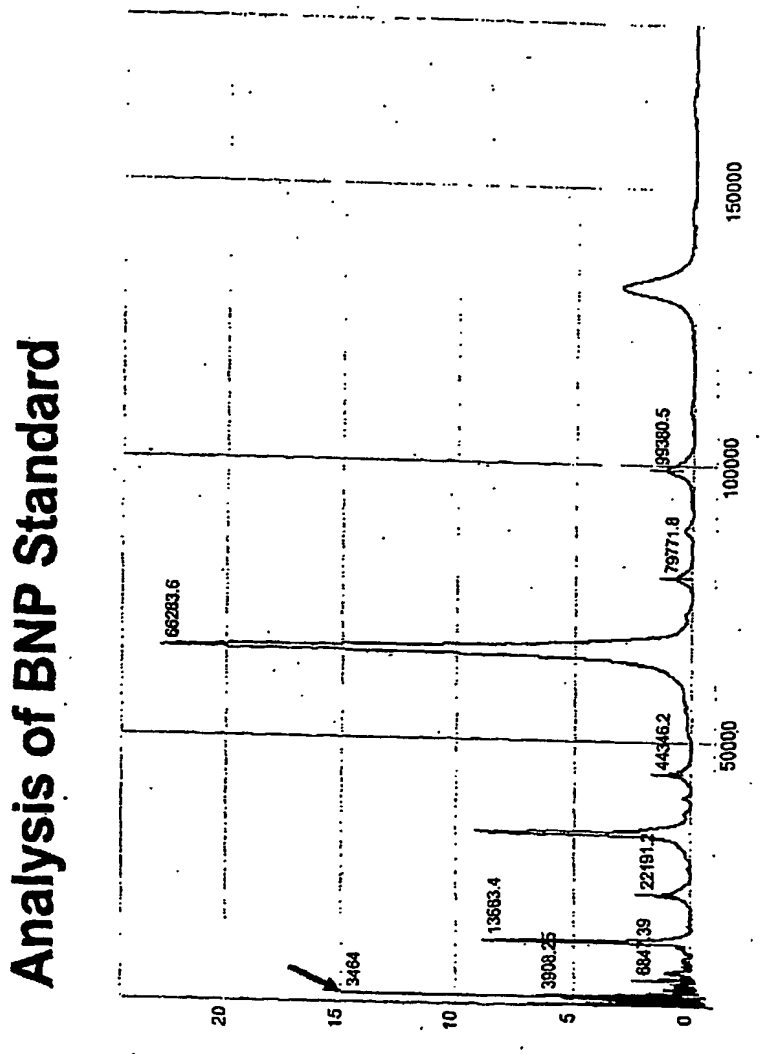


FIG. 2A

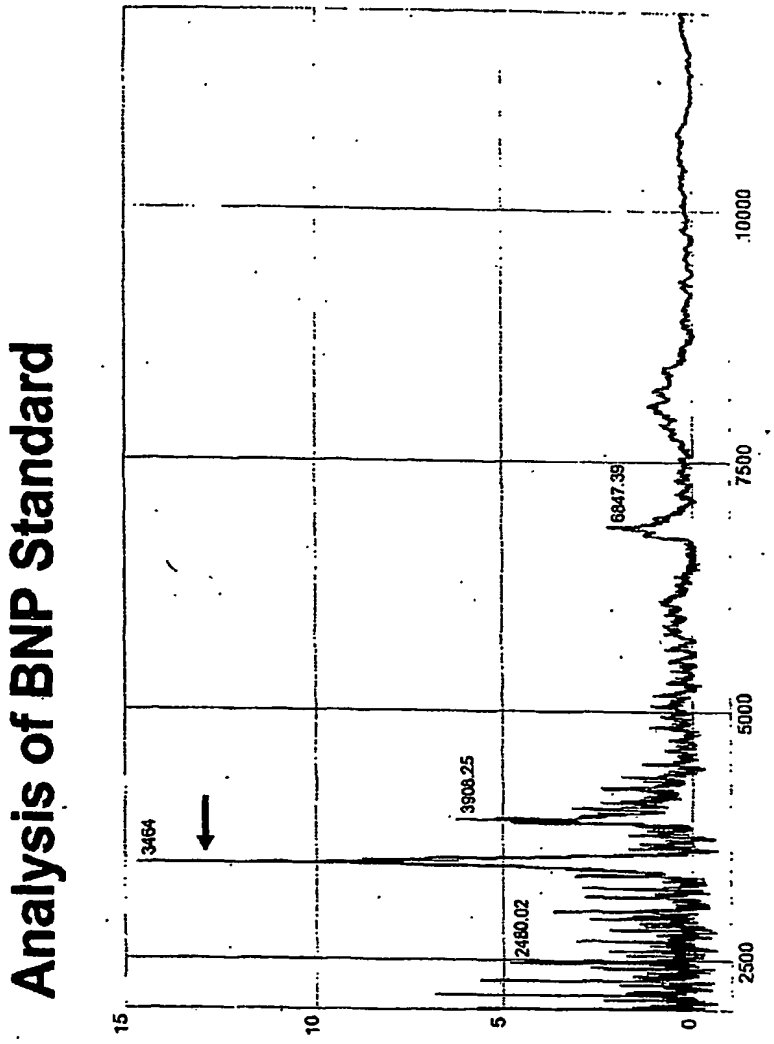
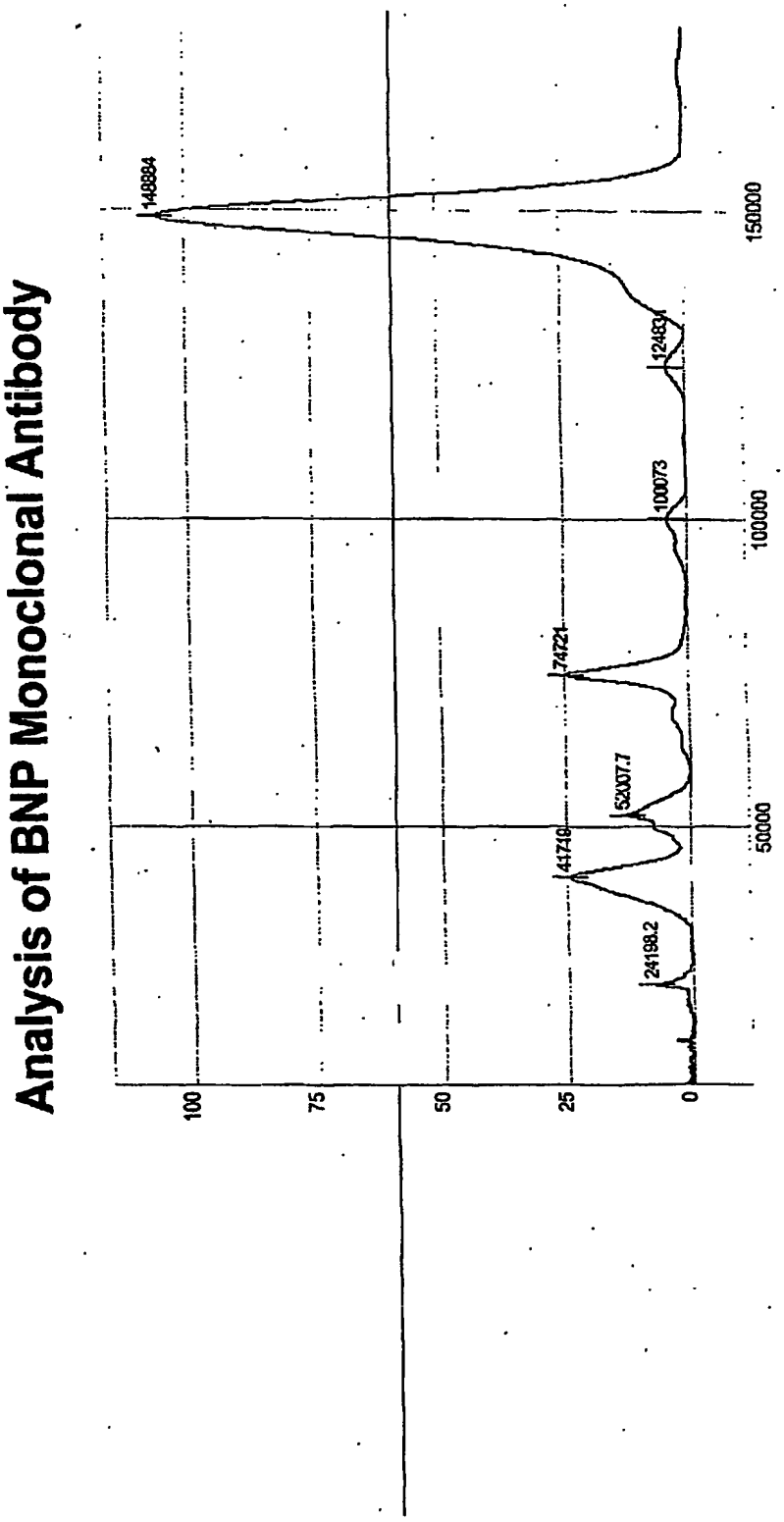


FIG. 2B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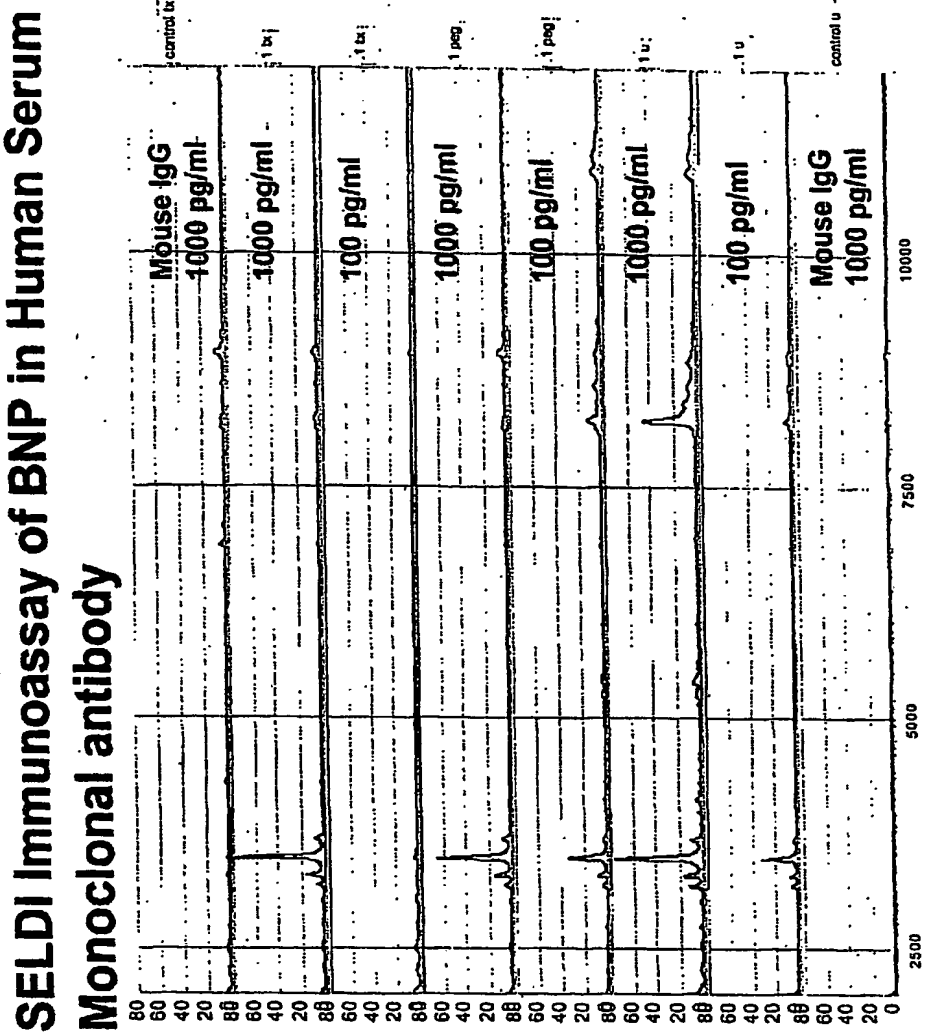
148884
124834
100073
74221
52007.7
41748
24198.2
150000
100000
50000
100
75
50
25
0

FIG. 3


SELDI Immunoassay of BNP in Human Serum
Monoclonal antibody
Urea CHAPS wash
PEG Tx PBS wash
Tx PBS wash
Mouse IgG 1000 pg/ml
1000 pg/ml
100 pg/ml
1000 pg/ml
100 pg/ml
1000 pg/ml
100 pg/ml
Mouse IgG 1000 pg/ml
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.1 tx
1 peg
.1 peg
1 u
.1 u
control u
2500
5000
7500
10000

FIG. 4A

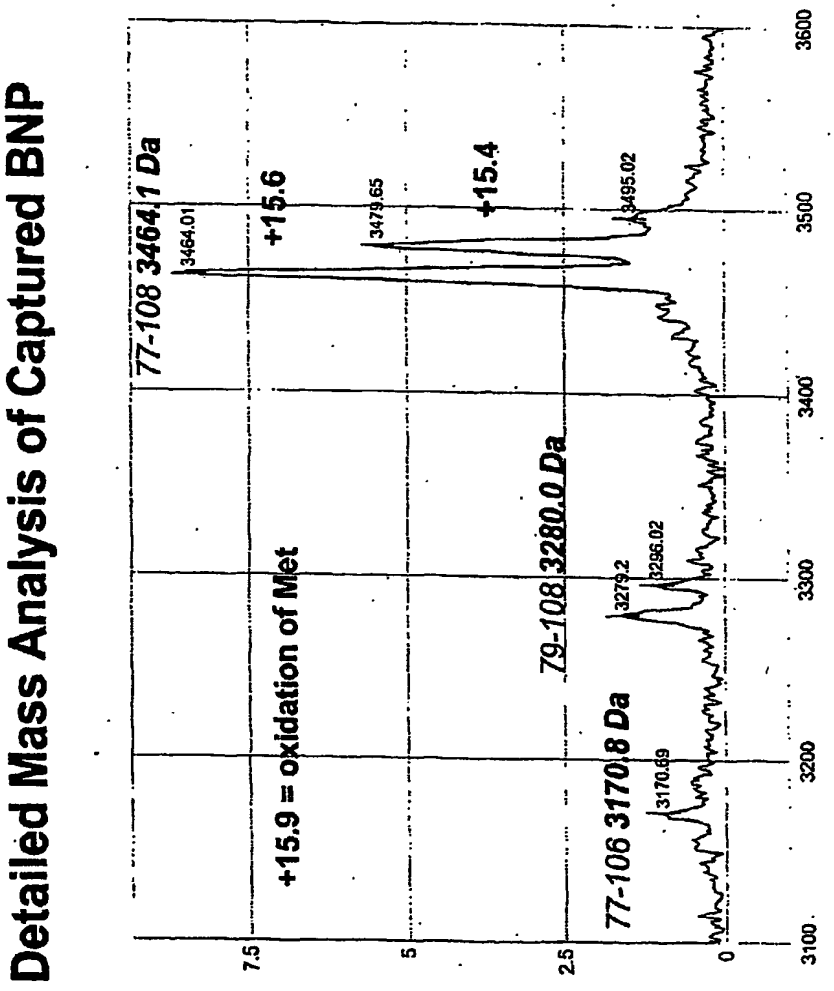
Detailed Mass Analysis of Captured BNP
77-108 3464.1 Da
+15.6
+15.4
3464.01
3479.65
3495.02
+15.9 = oxidation of Met
79-108 3280.0 Da
3279.2
3296.02
77-106 3170.8 Da
3170.69
7.5
5
2.5
0
3600
3500
3400
3300
3200
3100

FIG. 4B

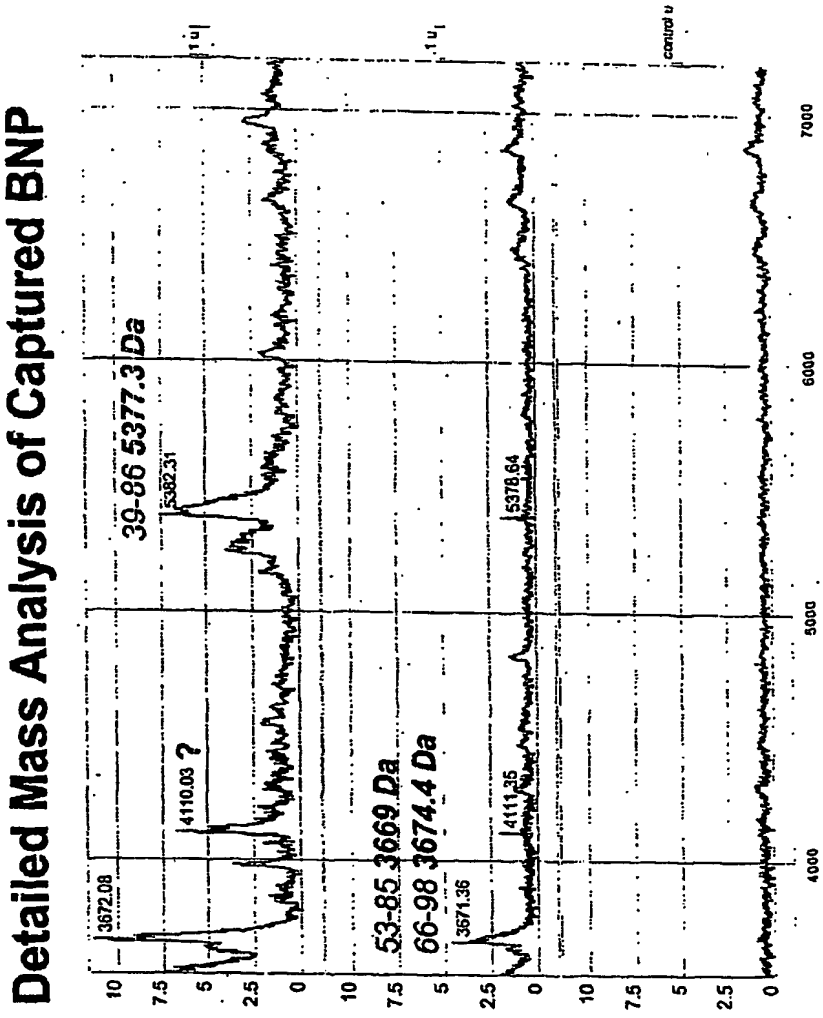
Detailed Mass Analysis of Captured BNP
39-86 5377.3 Da
53-85 3669 Da
66-98 3674.4 Da
3672.08
4110.03 ?
5382.31
3671.36
4111.35
5378.64
control u

FIG. 4C

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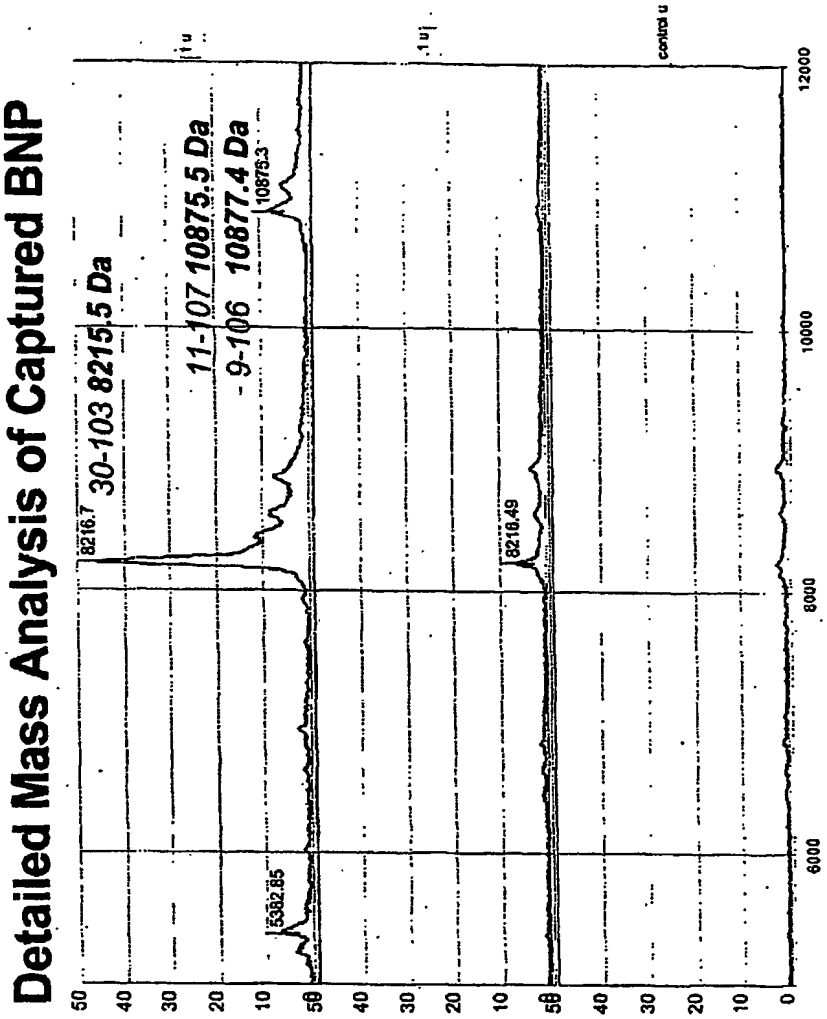

FIG. 4D

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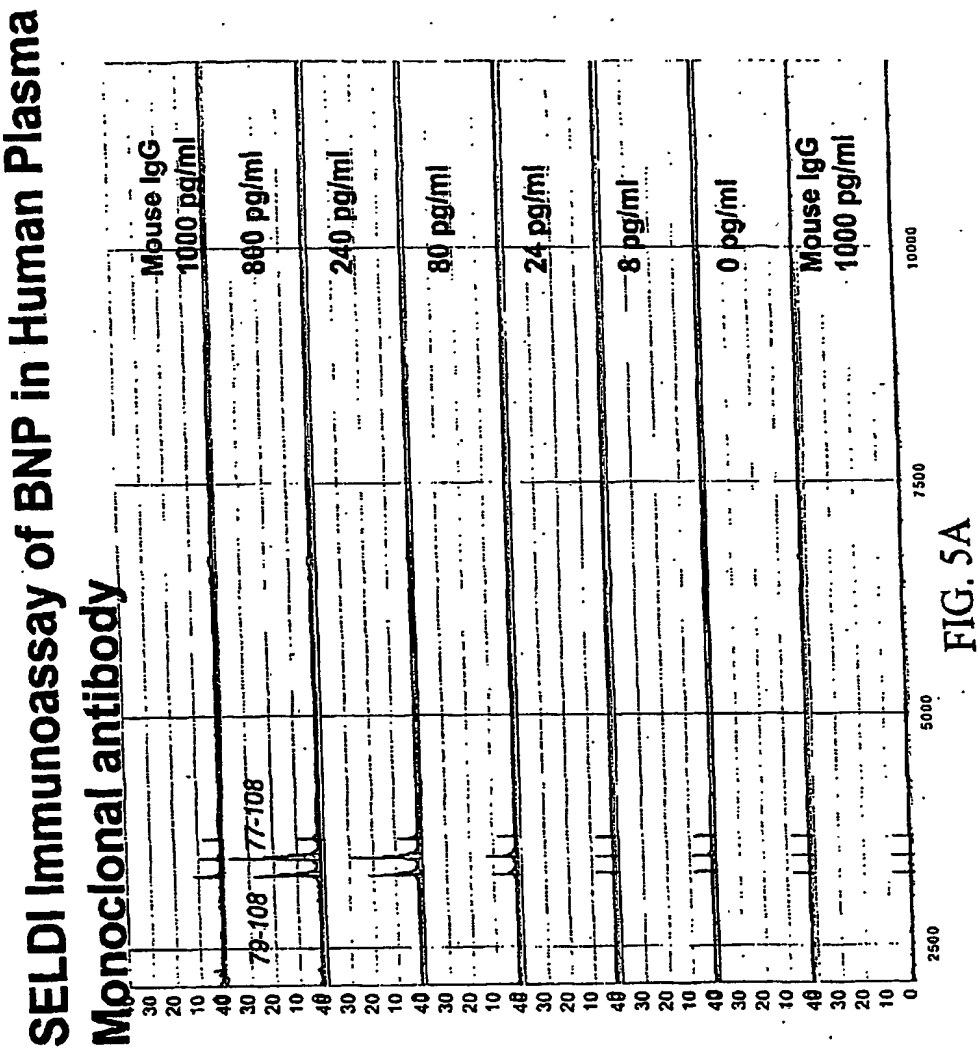

FIG. 5A

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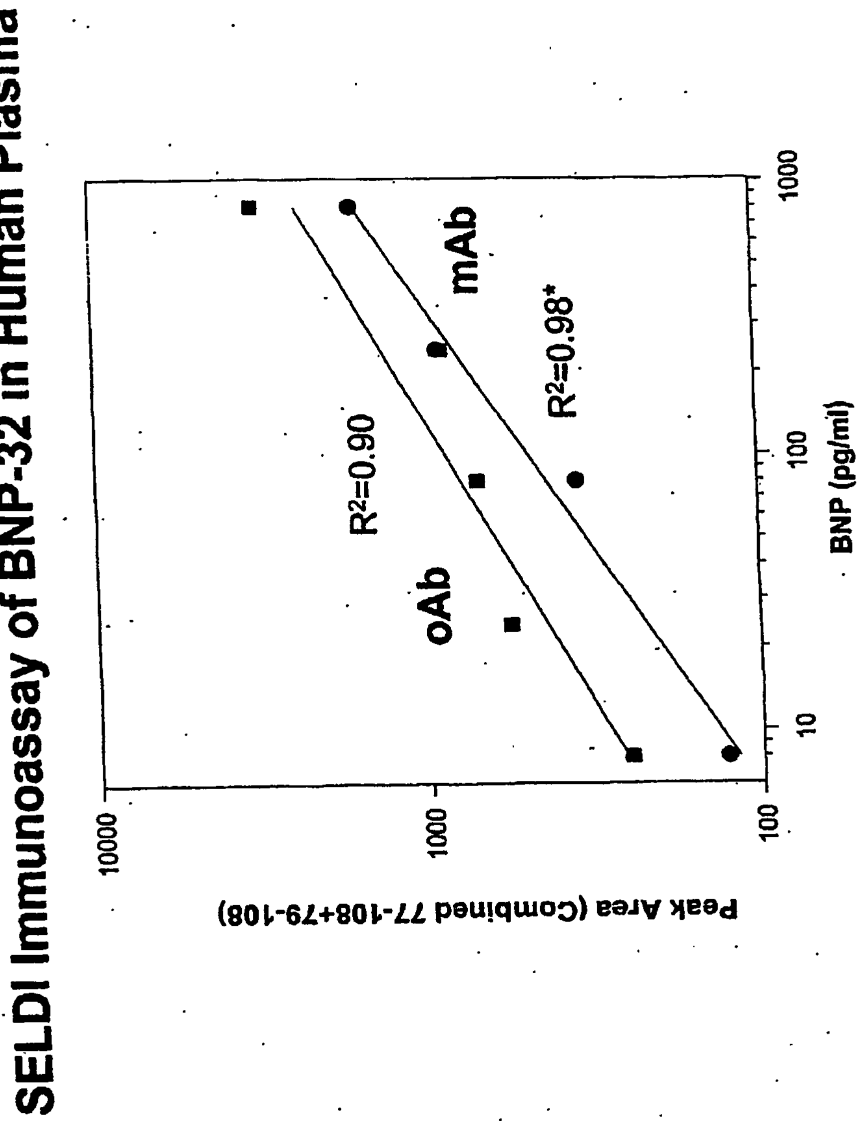

FIG. 5B

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App No.: Not yet assigned
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Attorney: Leslie E. Aberman
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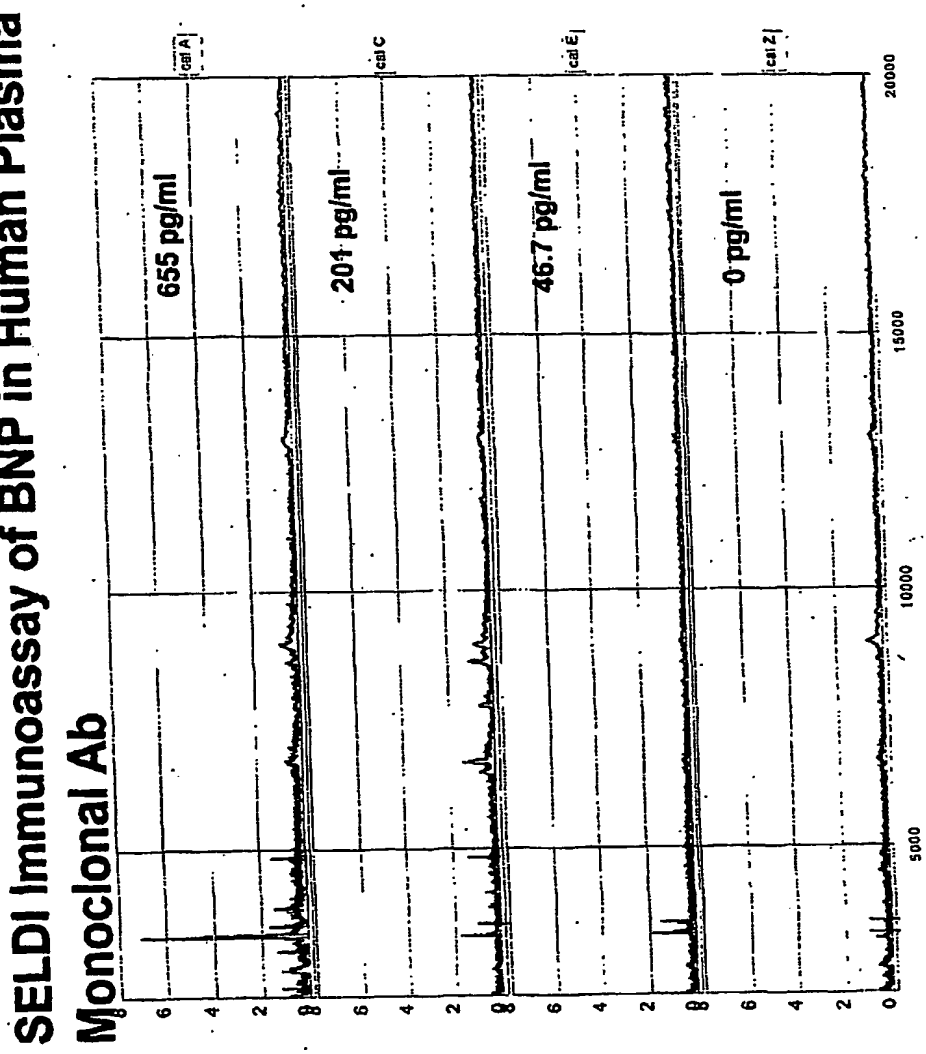

FIG. 6A

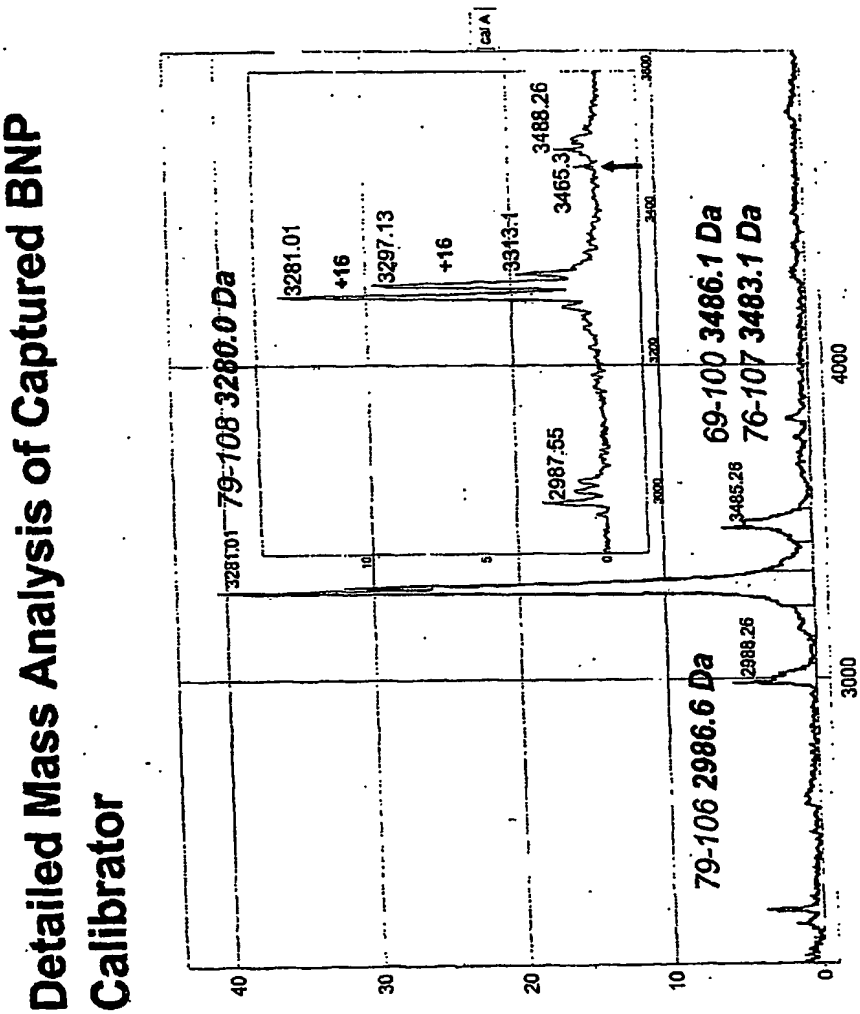
Detailed Mass Analysis of Captured BNP
Calibrator
Cal A
3281.01
+16
3297.13
+16
3313.1
3465.3
3488.26
2987.55
3281.01
79-108 3280.0 Da
69-100 3486.1 Da
76-107 3483.1 Da
3485.26
2988.26
79-106 2986.6 Da
40
30
20
10
0
3000
4000

FIG. 6B

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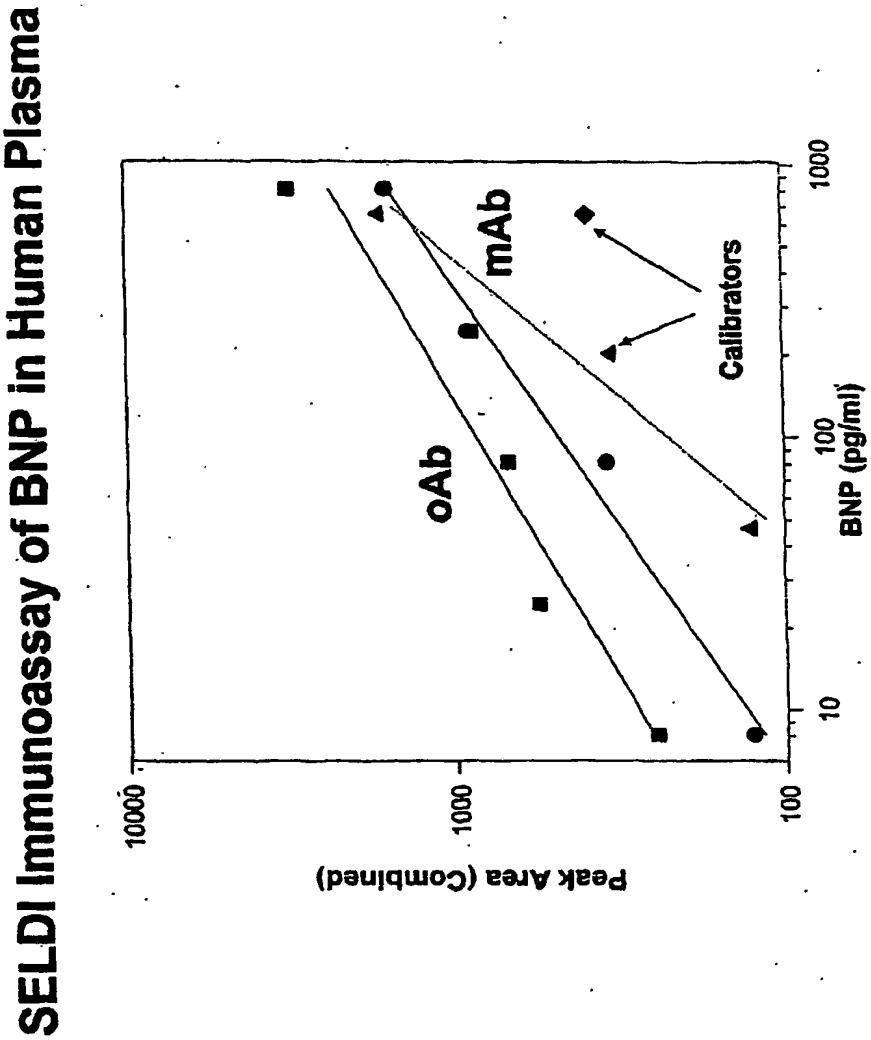

FIG. 6C

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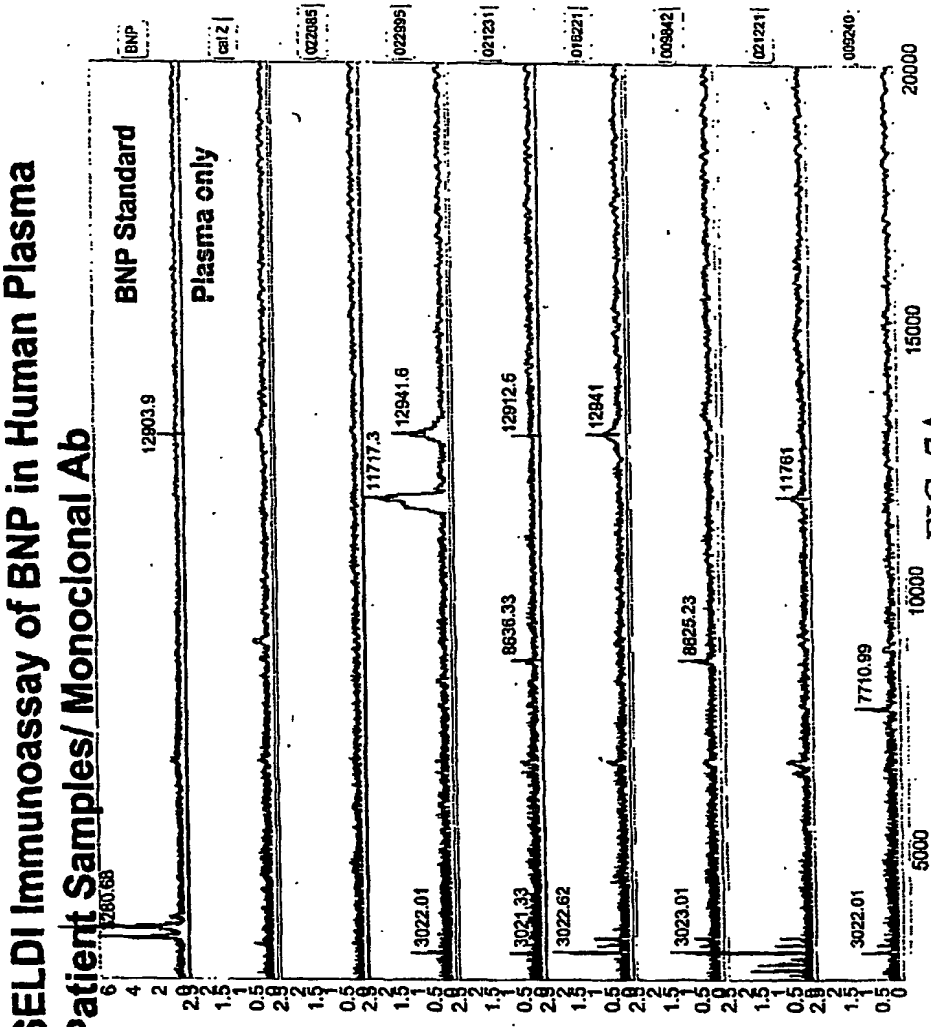

FIG. 7A

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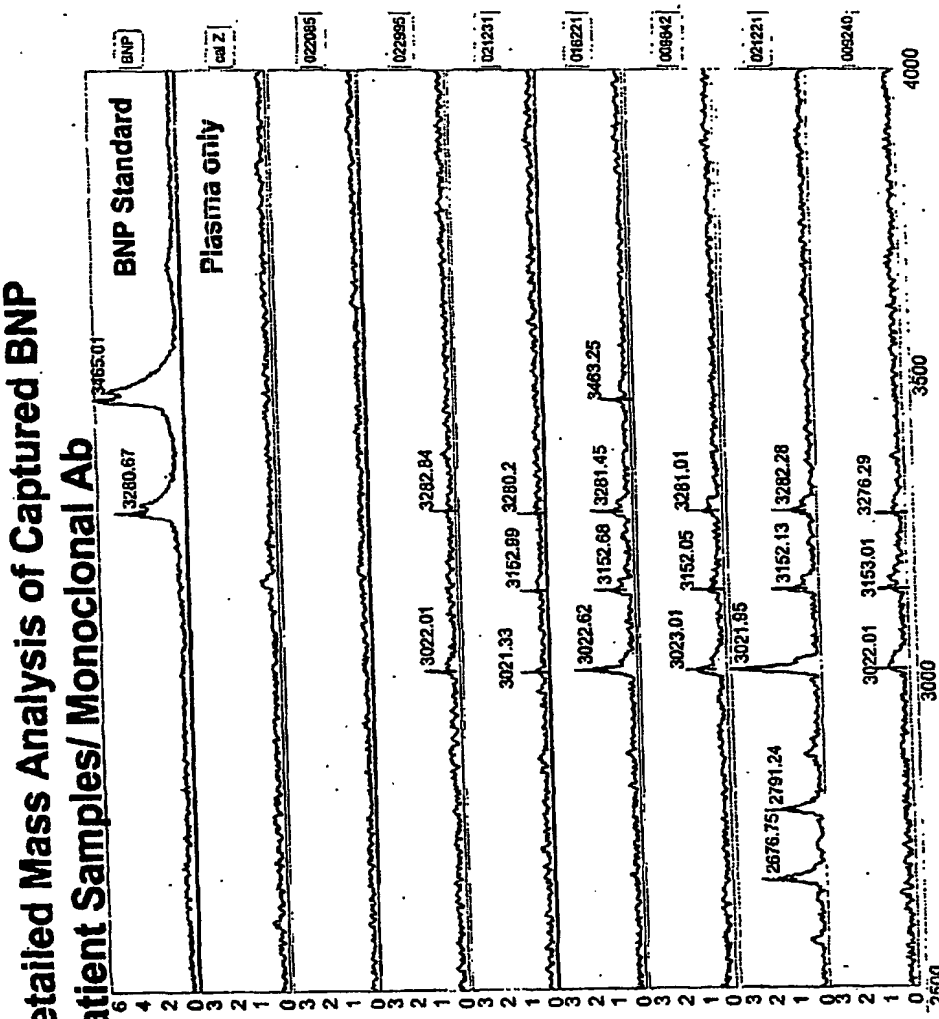

FIG. 7B